CARBON DIOXIDE TENSION AND SEXUAL DIFFERENTIATION IN HYDRA

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In the past few years an extensive literature on a "sex gas" (Loomis, 1959b) of *Hydra* has accumulated. Loomis (1954) reported results appearing (p. 146) "to indicate that the primary stimulus that induces sexual differentiation in *Hydra littoralis* is a critical lowering of the oxygen concentration of the medium." Loomis and Lenhoff (1956) next reported, however, that (p. 562) ". . . although reduced oxygen tension is generally found in sexual cultures, it is not the causative factor *per se*." The authors further stated (p. 562): "Attempts to maintain hydra in the asexual condition were found to demand (a) frequent changes of water, (b) uncrowded conditions or (c) extreme shallowness (2–3 mm)." These observations were in contrast to those of Park (1956) on mass cultures of a clone of *H. littoralis* maintained from 1950 to 1956, and also to the observations of Burnett (1961) on a mass culture of *H. oligactis*. Moreover, Park *et al.* (1961), using a clone of Loomis's stock of *H. littoralis*, found that uncrowded conditions were not sufficient to maintain the hydras in the asexual state.

Loomis (1957; see also 1959a, 1959b, 1959c, 1960 and 1961) next concluded that (1957, p. 738) ". . . sexual differentiation may be reversibly induced in *Hydra* by measures that control the pCO_2 of their aqueous environment." More recently and interestingly, however, Loomis (1959d, 1961) reported that sexual differentiation of *Hydra* could not be induced by measures that increase the pCO_2 of the aqueous environment unless some feedback was permitted between *Hydra* and this environment.

The lack of agreement between the reports cited above and those of Burnett (1961), Park (1956) and Park *et al.* (1961) make it important to investigate further the efficacy of increased environmental pCO_2 in inducing sexual differentiation of *Hydra*. It is of particular importance to test the effect of a number of different methods of controlling pCO_2, including Loomis's method (Loomis, 1957), on the Loomis stock of *Hydra*, as well as to test the effect of the Loomis method on other species of *Hydra*. The results of such tests are the subject of this report.

Materials and Methods

Three species of *Hydra* from different sources were used: (1) *H. littoralis*, (2) *H.* (sp.), and (3) *H. pseudoligactis*.

H. littoralis individuals were descended from a single mature male of Loomis's stock. Mass cultures of this clone have been maintained in our laboratory for four

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years. A brief description of the course of differentiation and subsequent disappearance of male gonads is given here to clarify some of the criteria used. The first indication of differentiation of gonadal tissue is a thickening of the ectoderm, usually in the subhypostomal region, though sometimes more proximally. The next day one sees, proximal to the site of the original thickening, discrete, mammiform protuberances of the ectoderm. These are the mature gonads, which during successive days move progressively nearer the budding region (just distal to the junction of body and stalk). The more proximal they are, the smaller and less mammiform they are. Those nearest the budding region often have a ragged appearance, and in the budding region appear as barely visible snags of tissue. Before the complete disappearance of the gonads in the budding region, the ectoderm of the subhypostomal region may again thicken and the whole process of differentiation, maturation, and disappearance of gonads may recur. Under oil immersion we have seen motile sperm in squashed preparations of mammiform gonads.

Individuals of the second species used were descended from a male purchased from the Carolina Biological Supply Company and believed to be *H. littoralis*. After completion of the experiments, however, the conclusion was reached (Dr. Helen Forrest, personal communication) that this is not *H. littoralis*, but a species as yet unreported in North America. The periodic sexual differentiation in mass laboratory cultures was similar to that found for the Loomis stock of *H. littoralis* (Park, 1961). This *Hydra* had been maintained in mass culture for 6 months prior to the first experiment.

The *H. pseudoligactis* individuals were descended from a culture obtained from the Gladstone, Oregon, laboratories of the Carolina Biological Supply Company. Mass cultures had been maintained in the laboratory for two months prior to the experiment. No sexual forms have been found in laboratory cultures which have now been maintained for two years.

Mass cultures of each of the three species have been maintained according to the methods of Loomis and Lenhoff (1956), at laboratory temperature (23° ± 2° C.). Unless otherwise noted, all animals selected at the start of each experiment were asexual and without buds. Selection was made under a stereoscopic microscope at 10 × magnification.

In all experiments, cultures were offered an excess of brine shrimp larvae once daily for one hour, cleaned and re-covered with a tap water solution of 100 mg./l. $NaHCO_3$, 50 mg./l. disodium salt of ethylenediamine tetra-acetic acid, and 50 mg./l. $CaCl_2$ (Ca-BVT), unless otherwise noted. Two culture situations were used: (1) 10 hydras per 15-ml. beaker containing 15 ml. culture solution. Newly dropped buds were removed just before daily feeding; (2) 100 hydras per finger bowl (63 mm. inside diameter) containing 40 ml. culture solution. Culture size was maintained by random discard during the daily cleaning.

Three methods of controlling environmental pCO_2 were used: (1) injection (Loomis, 1957), (2) bubbling gas mixtures through cultures, and (3) flow of gas mixtures over cultures.

The injection method of increasing the pCO_2 of cultures consists of injecting cultures with solution that has previously been shaken in a syringe with gas mixtures of varying percentages of CO_2 and O_2. The source of water used for the solution was not stated by Loomis in 1957, but Loomis and Lenhoff (1956), described it as

tap water, which we also used. Instead of following the procedure described by Loomis in 1957 (p. 736) for exposing solution to 100% O_2, "The culture solution was shaken three times before use with a large excess of 100 per cent oxygen . . .," we bubbled 100% O_2 vigorously through one liter of solution in a two-liter suction flask for 20 minutes. The flask was then closed tightly and the solution drawn off as needed.

In the bubbling method, a gas mixture from a pressure cylinder flowed through rubber tubing into a medium porosity, ceramic filter stick placed beneath the surface of the solution in the culture vessel. Carbon dioxide-free air for controls was obtained by pumping room air through 20% KOH.

In the flow method, a gas mixture flowed from a pressure cylinder or an air pump at 75 cc. per minute into the stem of an inverted funnel suspended over a culture dish. There was a $\frac{1}{8}$-inch space between the funnel and the rim of the dish.

Experiments and Results

(1) *Injection*

(a) Loomis stock

The injection procedure described in detail by Loomis (1957) was used on 10-hydra cultures twice daily for 14 days with 5.6%, 5% and 1.7% CO_2 in O_2, and 100% O_2. The experiments were done on four occasions over 18 months, each time comparing a single concentration of CO_2 with 100% O_2 and twice also with controls exposed to room air. The results are shown in Figure 1.

Although the four experiments yielded a diversity of results, a few general statements may be made: (1) Hydras began to differentiate sexually within 24 hours, regardless of treatment. (2) The percentage of sexual forms increased for 2–4 days, then decreased in varying degrees. (3) The decreases ranged from small in the 5.6% CO_2-treated cultures of experiment I to large in the 100% O_2-treated cultures of experiment III. (4) While exceptions were observed (notably in experiments I and III), the percentages of sexual forms tended to rise and fall together within an experiment regardless of treatment.

(b) *H.* (sp.) and *H. pseudoligactis*

Injection was used *once* daily for 18 days on 10-hydra cultures of *H.* (sp.) and *H. pseudoligactis*. Five cultures of *H.* (sp.) and 4 of *H. pseudoligactis* were injected with calcium-free BVT that had been exposed to 5.6% CO_2 in O_2, 5 cultures of *H.* (sp.) were in the solution flushed with 100% O_2, and 5 cultures of *H.* (sp.) and 4 of *H. pseudoligactis* remained open to room air. No *H. pseudoligactis* individuals became sexual. No *H.* (sp.) individuals in cultures injected with CO_2-equilibrated solution became sexual; one in solution flushed with 100% O_2 was sexual from day 7 to day 13, and five exposed to room air were sexual from day 9 to day 13.

The experiment was repeated on *H.* (sp.) a year later. This time no sexual differentiation occurred under any of the treatments.

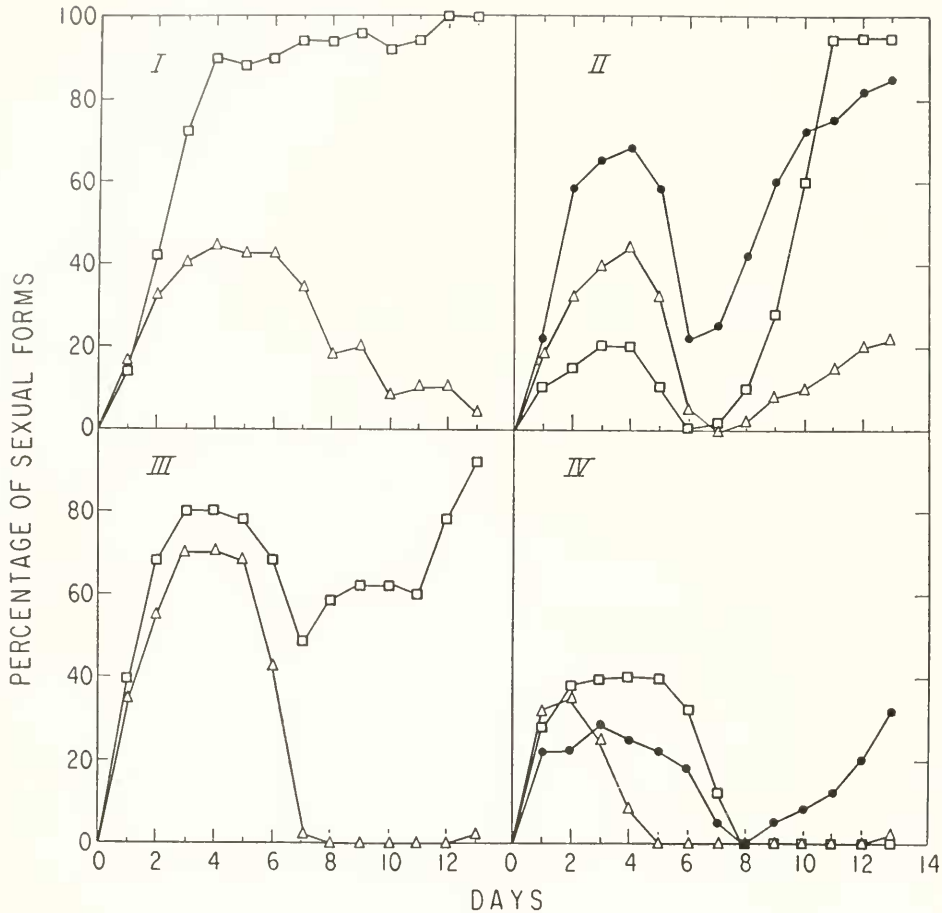

FIGURE 1. Four experiments showing percentage sexual forms in 10-hydra cultures exposed by the Loomis injection method twice daily to CO_2 (□) (5.6% in I and II, 5.0% in III, and 1.7% in IV), or to 100% O_2 (△), or to room air (●). Fifty hydras per curve in I, 40 in II, III and IV.

(2) Bubbling (Loomis stock)

(a) 5.0% CO_2 in air *vs.* CO_2-free air

One hundred-hydra cultures in calcium-free BVT were bubbled 22 hours daily for 40 days, one with 5% CO_2 in air, another with CO_2-free air. The second and third times this experiment was done a third culture was exposed to standing room air. The results are shown in Figure 2.

As in the injection experiments, hydras under *all* experimental conditions began to differentiate sexually within 24 hours. In cultures bubbled with CO_2-free air, as well as in those bubbled with 5% CO_2 in air, the percentage of sexual forms reached a maximum between days 4 and 6, then decreased rapidly. Cultures bubbled with 5% CO_2 in air showed a second increase in percentage of sexual

forms, but even under this condition of elevated pCO_2, the percentage decreased again to zero in two replicates and remained below 15% in the third. A high, though variable, percentage of sexual forms in each of the undisturbed cultures in room air persisted from day 10 until the end of the experiment.

(b) Effect on sexual forms

Loomis (1959a) reported that (p. 266) ". . . hydra *de*differentiated to the asexual state a few days after turning on the aerator of the aquarium." To test the effect of bubbling on cultures containing both sexual and asexual hydras, the preceding experiment was slightly modified and was done three times. (1) Hydras were taken at random from the stock cultures so that on zero day, the experimental

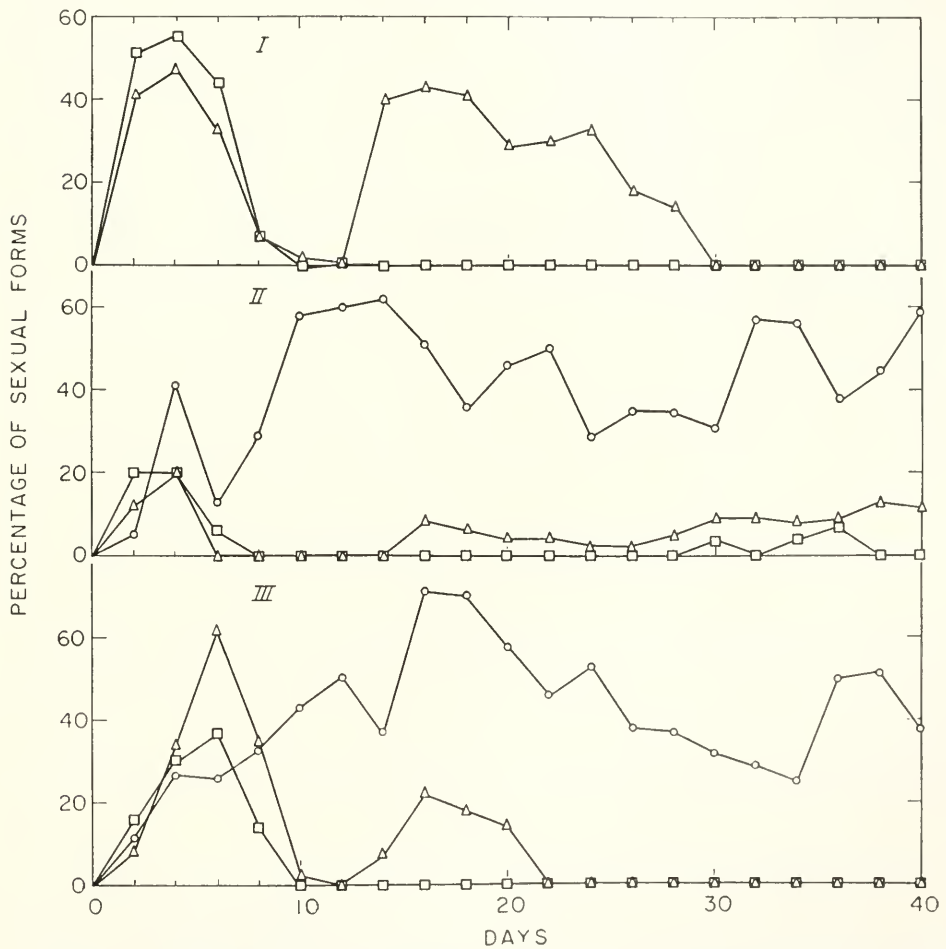

FIGURE 2. Three experiments showing percentage sexual forms in 100-hydra cultures bubbled 22 hours daily with 5% CO_2 in air (△), CO_2-free air (□), or exposed to room air (○).

cultures contained both sexual and asexual forms; (2) room air was used instead of CO_2-free air, and (3) the culture standing in room air was omitted. One replicate experiment was terminated on the fourteenth day. The results of the two 40-day replicates are shown in Figure 3.

In cultures bubbled with 5% CO_2 in air, as well as in those bubbled with room air, there was an immediate and rapid decrease in percentage of sexual forms. In the replicate experiment run 14 days, sexual forms in the 5% CO_2 bubbled culture decreased from 71% to 0 by day 10. In the culture bubbled with room air the decrease was 72% to 0 by day 12. It will be noted that one culture bubbled with 5% CO_2 in air remained asexual for the last 28 days of the experiment. In

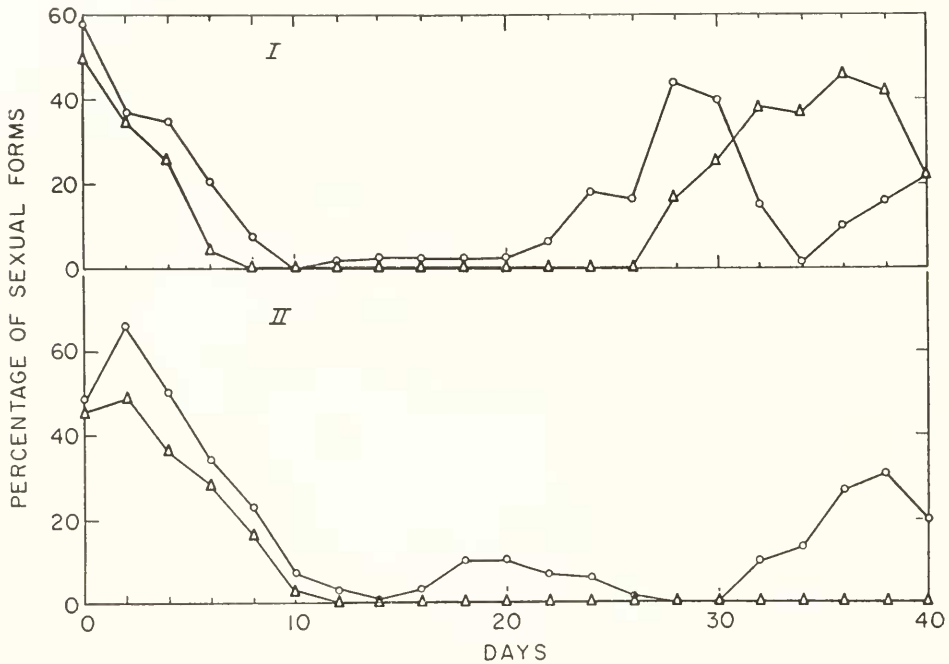

FIGURE 3. Two experiments showing percentage sexual forms in 100-hydra cultures bubbled 22 hours daily with 5% CO_2 in air (△), or room air (○). Both sexual and asexual forms on zero day.

general, bubbling with 5% CO_2 was as effective as bubbling with room air in maintaining the hydras in an asexual condition.

(c) Bubbling two hours *vs.* a single injection daily

An experiment was done on 10-hydra cultures to compare bubbling two hours daily with injection once daily for 20 days. Four cultures in calcium-free BVT were bubbled with 5% CO_2 in O_2, four were injected with calcium-free BVT that had been equilibrated with 5.6% CO_2 in O_2, and four cultures remained undisturbed in room air between successive feeding and cleaning operations. When the experi-

ment was repeated, four additional cultures were bubbled with 5% CO_2 in air. The results are shown in Figure 4.

Under all treatments hydras began to differentiate sexually within three days. In cultures bubbled with 5% CO_2 in O_2, the percentage of sexual forms reached a maximum by the fourth day, then decreased through the eighth day, and remained at less than 10 for the remaining 10 days of the experiments. In general, with the notable exception of a low percentage of sexual forms in the 5% CO_2-bubbled

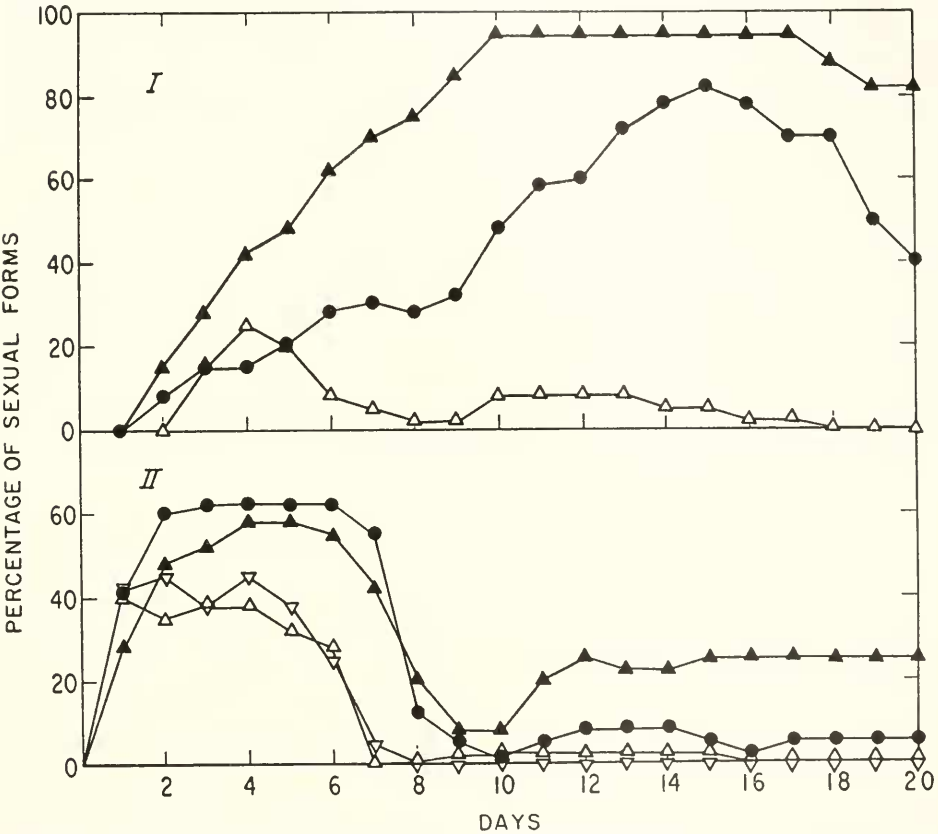

FIGURE 4. Two experiments showing percentage sexual forms in 10-hydra cultures injected once daily with 5.6% CO_2 in O_2-equilibrated solution (▲), or bubbled two hours daily with 5% CO_2 in O_2 (△), or 5% CO_2 in air (▽), or exposed to room air (●). Forty hydras per curve.

cultures throughout experiment I, there was considerable similarity in the results of the different treatments within each experiment.

In experiment II, early sexual differentiation was quickly followed by disappearance of gonads under all treatments. By the tenth day there were almost no sexual forms in any of the cultures. As in the experiments with injection twice daily (see Figure 1), the percentages of sexual forms again tended to rise and fall

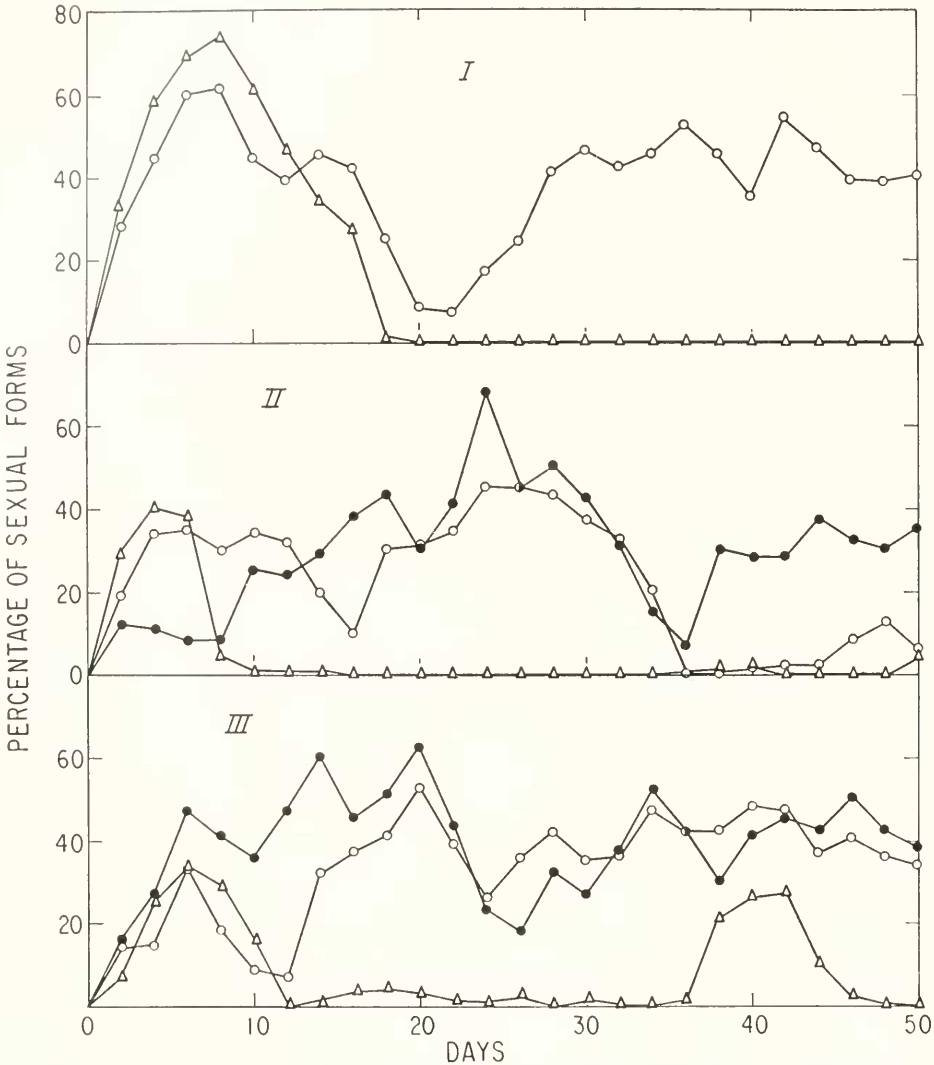

FIGURE 5. Three experiments showing percentage of sexual forms in 100-hydra cultures exposed to 5% CO_2 in air (△), or CO_2-free air (○), by flowing gas mixtures over the cultures. In two experiments a third culture was open to room air (●).

together, regardless of treatment. It is of interest that in the group of 4 cultures treated with CO_2 by injection, all of the sexual forms from day 13 to day 20 were in one culture while the other three cultures remained asexual.

(3) Flow of gas mixtures (Loomis stock)

One 100-hydra culture in calcium-free BVT, under a funnel, was exposed to 5% CO_2 in air and a comparable culture was exposed to CO_2-free air 22 hours

daily for 50 days. The experiment was done three times. The last two times an additional 100-hydra culture was left open to room air. The results are shown in Figure 5.

Again, consistent with early sexual differentiation during other types of exposure, gonads began to develop within 24 hours in cultures under flowing gases, regardless of the mixtures used. Under CO_2 in air there was an increase in percentage of sexual forms for 4–8 days, followed by a rapid return to zero. There was almost no sexual differentiation for the following 24–40 days. By contrast, when cultures were exposed to CO_2-free air, large, though variable, percentages of sexual forms persisted throughout most of the experimental period. In each of the last two replicates, the percentages of sexual forms in cultures exposed to CO_2-free air were about the same for long periods as in cultures standing open to room air.

Discussion

Asexual Loomis stock hydras developed gonads within a few days, regardless of whether the culture solution had a low pCO_2 (saturated with 100% O_2, CO_2-free air, or room air) or high pCO_2 (equilibrated with 1.7%, 5.0%, or 5.6% CO_2). We can, therefore, conclude that sexual differentiation in these hydras was not controlled by controlling the pCO_2 of the aqueous environment, within the limits of these experiments.

Loomis stock hydras were exposed to culture solution in which the pCO_2 was raised and maintained high by methods (continuous surface gassing and continuous bubbling) different from, and at least as efficient as, the injection method. In such experiments, essentially no new sexual differentiation occurred for as long as 30 days after the initial temporary gonad development. Elevated pCO_2 cannot, therefore, be said to promote or maintain sexuality under these conditions.

Furthermore, since no hydras of either the unidentified species or of *H. pseudoligactis* differentiated sexually when cultures were injected once a day with solution exposed to 5.6% CO_2, we can conclude that increasing the pCO_2 of the aqueous environment to this level does not induce sexual differentiation in these species.

Coupled with the results of our investigations, the progressive modifications by Loomis of the "sex gas" theory of sexual differentiation of *Hydra* present a number of aspects that merit discussion.

First, mention should be made of the wide variety of conditions in the laboratory under which the Loomis stock of *H. littoralis* differentiates sexually. In addition to sexual differentiation under environmental CO_2 tensions from zero to 5.6%, using three methods of controlling pCO_2, we have found that this stock may turn sexual in population densities ranging down to one hydra isolated in 10 ml. calcium-free BVT (Park *et al.*, 1961). Moreover, we have found that this stock differentiates sexually as readily and as often in a dilute salt solution (in double distilled water, the last distillation from glass) which contains neither bicarbonate nor ethylenediamine tetraacetic acid, as it does in calcium-free BVT (Park, 1961).

A second relevant point concerns time of sexual differentiation of *Hydra* in nature. Sexual forms of a number of North American species have been found in the *spring* of the year (Downing, 1909; Forrest, 1959, 1963; Griffin and Peters, 1939; Hyman, 1931; Trowbridge *et al.*, 1936; Whitney, 1907) from which we can

conclude that the statement ". . . *Hydra* . . . turn sexual in the late fall . . ." (Loomis, 1961, p. 358) is an incautious generalization. Moreover, *H. littoralis*, the species on which the work leading to the "sex gas" theory was done, has been found sexual in March (Trowbridge *et al.*, 1936) as well as in the *fall* (Hyman, 1931). More recently, Forrest (personal communication) reported that she has collected sexual forms of *H. littoralis* in nature from late February to mid-October, the time of year varying with the locality.

A third point concerns the induction of sexual differentiation in certain clearly specified species of *Hydra* by lowering the temperature (see Brien, 1961, and Hyman, 1928 for reviews). It has been implied that stagnation had more to do with sexual differentiation of hydras in refrigerators than did lowered temperature (Loomis, 1959b). Nevertheless, the reports of experiments on *H. oligactis* (Brien and Reniers-Decoen, 1949); on *Pelmatohydra robusta* (Itô, 1954); on *H. parva* (Itô, 1955); and on *H. pirardi* (Brien, 1961; Burnett, 1961) clearly show that the refrigerated cultures that became sexual received the same care as the controls at higher temperatures and that the controls did not become sexual. We have confirmed the results on *H. oligactis* and *H. pirardi* and have also obtained similar results on *H. pseudoligactis*.

Finally, any theory attempting to explain the onset of sexual differentiation—or the onset of any other biological event—under laboratory conditions, ought, it seems to us, to take account of what is known about these biological events in nature. A case in point is the information available on sexuality in *H. littoralis* in its natural habitats. Three reports, Hyman (1931, 1938), Trowbridge *et al.* (1936) and Forrest (personal communication), state that sexual forms of this species were found in *swiftly* moving water. These observations show that sexual differentiation can occur where feedback between a hydra and its environment would be expected to play a minor role.

It seems safe to say that the inductor of sexual differentiation of *Hydra* either in nature or in the laboratory is still to be sought.

Summary

1. One hundred- and 10-hydra cultures of *Hydra littoralis* and 10-hydra cultures of *H. pseudoligactis* and *H.* (sp.) were maintained 14–50 days in culture solutions equilibrated with gas mixtures varying in CO_2 content from 0.0% to 5.6%.

2. Two culture solutions and three methods of gassing cultures were used.

3. The results were:

   a. Asexual *H. littoralis* began to differentiate sexually within 1–4 days under all experimental conditions; initial sexual differentiation was temporary and was often followed by a second period of gonad development. In several experiments, percentages of sexual hydras tended to rise and fall together, regardless of treatment.

   b. *H. littoralis* cultures containing 46–72% sexual animals bubbled 22 hours daily with room air or 5% CO_2 in air were 95–100% asexual by day 12. Continued bubbling for the next 4 weeks with 5% CO_2 in air was as effective as bubbling with room air in maintaining asexuality.

c. No sexual differentiation occurred in *H. pseudoligactis* or *H.* (sp.) cultures exposed 18 days to elevated pCO_2. There was no sexual differentiation in *H. pseudoligactis* cultures exposed 18 days to room air. However, 5% of *H.* (sp.) in room air were sexual from day 9 to day 13.

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